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## Abstract

Laminin-rich basement membrane (BM) guides epithelial cell polarity, regulates epithelial cell behavior and maintains the integrity of epithelial tissues.  $\alpha\beta_1$ - and  $\alpha_6\beta_4$ -integrins both contribute to laminin adhesion and signaling via the assembly of integrin adhesion complexes that help to orient the apico-basal polarity axis.  $\beta_4$ -integrin differs from other integrin subunits due to its large cytoplasmic domain that connects to cellular intermediate filament (IF) networks in specialized adhesions called hemidesmosomes (HD).  $\beta_4$ -integrin is only known to form a heterodimer with the  $\alpha_6$ -subunit. In normal tissues,  $\beta_4$ -integrin is expressed in cells that also express the  $\alpha_6$ -subunit. However, in most cells analyzed,  $\beta_4$ -integrin is expressed in large excess over  $\alpha_6$ -integrin and in some tumor cells,  $\beta_4$ -integrin appears to promote tumorigenic signaling despite loss of HDs formation. The fate of free  $\beta_4$ -subunit and its potential functions in cells have not been extensively studied. Here, we have studied subcellular localization and potential surface delivery of  $\beta_4$ -integrin in the absence of its heterodimer partner  $\alpha_6$ . We provide evidence that a significant fraction of  $\beta_4$ -subunit can reach the cell surface without  $\alpha_6$ -subunit. We also report that  $\beta_4$  is cleaved at its extracellular domain to produce a membrane-bound proteolytic product with an intact cytoplasmic domain. The processed  $\beta_4$ -integrin did not co-precipitate with  $\alpha_6$ -subunit. Taken together, our data suggest that  $\beta_4$ -integrin might have functions that are independent of heterodimer formation.

## Introduction

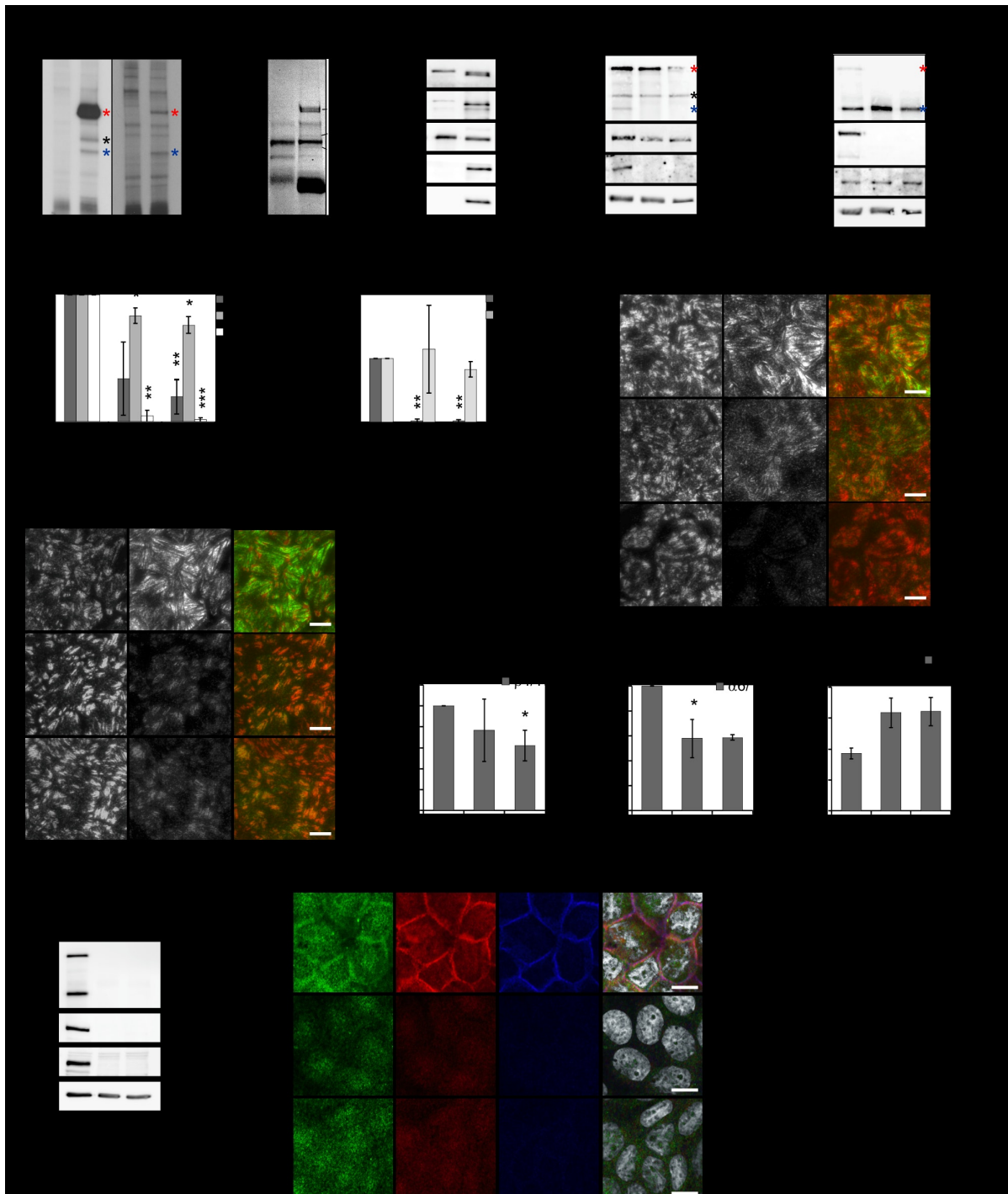
$\alpha_6\beta_4$ -integrin is an epithelial-specific heterodimeric extracellular matrix (ECM) receptor that binds select laminin isoforms of the epithelial basement membrane (BM) and forms the core of hemidesmosomes (HDs) [1] [2].  $\beta_4$ -integrin deviates from other integrins due to its large cytoplasmic domain that is responsible for binding to intermediate filaments (IFs) via plakins [3]. HDs establish mechanically robust connections between the ECM and cellular IF networks [4]. Together with laminin-binding  $\beta_1$ -integrins, such as  $\alpha_2\beta_1$ ,  $\alpha_3\beta_1$  and  $\alpha_6\beta_1$ ,  $\alpha_6\beta_4$ -integrins convey signals from the BM to regulate epithelial cell polarity [5] [6]. Although all of these integrins bind to laminin, they are only partially functionally redundant. Integrins have specific signaling roles that are critical to their functions in epithelial cells [7] [8] [9].

The intracellular domain of  $\beta_4$  possesses unique binding properties that are regulated on a different basis compared to other  $\beta$ -integrins subunits. Recent study suggests that  $\alpha_6\beta_4$  favors an extended conformation that might render it constitutively signaling competent [10]. It is now understood that the binding properties of the  $\beta_4$ -tail are regulated by phosphorylation of the  $\beta_4$ -tail in response to growth factor signaling [11]. Studies with chimeric receptors have shown that the cytoplasmic tail of  $\beta_4$  can support HD interactions and engage in signaling in the absence of the extracellular domain, which is required for heterodimerization with  $\alpha_6$  [12] [13] [14] [15]. Ligand-binding can also occur in the absence of the intracellular domain interactions [16] [17] [18], suggesting that the intra- and extracellular domains of  $\beta_4$  can indeed be functionally uncoupled. Earlier studies have shown that  $\beta_4$ -subunit forms heterodimers only with the  $\alpha_6$ -subunit, but is detected in excess over the  $\alpha_6$ -subunit, also at the cell surface [19] [20]. Based on pulse-chase analysis of integrin  $\alpha_6$  immunocomplexes, it was assumed that monomeric  $\beta_4$ -subunit cannot be translocated to the cell surface and thus the free  $\beta_4$ -subunit at the cell surface must derive from the dissociated  $\alpha_6\beta_4$ -heterodimer. However, as free inte-

grin  $\beta_4$  cannot be expected to co-precipitate with the  $\alpha_6$ -subunit, these results do not exclude the possibility that  $\beta_4$  integrin can be translocated to the cell surface as a single subunit. Interestingly, Klinowska et al. reported that  $\alpha_6$ -integrins were not required for early mammary gland development and demonstrated a presence of HD-like adhesions in mammary epithelial cells lacking  $\alpha_6$ -integrin [21]. In contrast, a more recent study found that  $\beta_4$ -integrin expression is essential for mammary gland development [22]. Taken together, these findings suggest that  $\beta_4$ -integrin might have functions that are independent of the formation of  $\alpha_6\beta_4$ -heterodimer.

### Objective

Given the possible  $\alpha_6$ -integrin-independent cellular function of  $\beta_4$ -subunit, we have here revisited this topic by studying the expression and localization of  $\beta_4$ -integrin in  $\alpha_6$ -integrin knockout ( $\alpha_6$ KO) MDCK cells. This study has important implications for the cellular role of  $\beta_4$ -integrins because monomeric  $\beta_4$ -subunit could potentially be signaling-competent.



a

### Figure Legend

**Figure 1. Integrin  $\beta_4$  subunit can reach the cell surface independently of the integrin  $\alpha_6$  subunit.**

(A) Metabolically labeled proteins co-precipitating with endogenous integrin  $\alpha_6$  and  $\beta_4$  in MDCK cells were resolved by SDS-PAGE in reducing conditions and visualized by autoradiography. Non-specific IgG was used for control immunoprecipitations.

(B) Coomassie staining of integrin  $\beta_4$  ( $\beta_4$ ) and non-specific IgG (IgG) co-precipitating proteins.

(C) Validation of the surface biotinylation method by Western blotting for selected markers for endosomes (EEA1), plasma membrane (Ecad), endoplasmic reticulum (Caln) and cytoplasmic microtubules ( $\beta$ -tub).

(D) Detection of surface expression of integrin  $\beta_4$  subunit in control and  $\alpha_6$ KO MDCK cells and (E) integrin  $\alpha_6$  subunit in control and  $\beta_4$ KO MDCK cells.

(F-G) Quantification of surface expressed integrins in  $\alpha_6$ KO (F) and  $\beta_4$ KO (G) cells by densitometry (n=3 experiments).

(H-I) TIRF imaging of talin (red) co-immunostained with integrin  $\beta_4$  (green) in control and  $\alpha_6$ KO cells (H) and with integrin  $\alpha_6$  (green) in control and  $\beta_4$ KO cells (I).

(J-K) Fluorescence intensity measurements of integrin staining from TIRF images. Intensity ratios were determined relative to talin signal intensity (15–17 images from 3 experiments were analyzed).

(L) TIRF-microscopy based co-localization analysis of integrin  $\alpha_6$  and talin in control and  $\beta_4$ KO cells was determined by measuring Pearson's correlation coefficients (13 images from 3 experiments were analyzed).

(M) Surface expression levels of  $\alpha_6$ -integrin in surface biotinylated control and  $\beta_1/\beta_4$ -dKO cells were analyzed by streptavidin-HRP blotting of  $\alpha_6$ -immunoprecipitations. Depletion of  $\beta_1$ - and  $\beta_4$ -integrins was confirmed by western immunoblotting of whole cell lysate.

(N) Confocal images of co-immunostained  $\beta_4$ - (green),  $\alpha_6$ - (red) and  $\beta_1$ -integrins (blue) in control and in the two  $\beta_1$ KO cell lines. A confocal slice at the lateral cell membrane is shown.

Statistical significance of fold changes (F-G & J-K) was tested with two-tailed one sample t test and PCC values (L) with one-way analysis of variance using Tukey's post hoc correction (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001). Scale bars = 10  $\mu$ m.

## Results & Discussion

To measure the levels of  $\beta_4$ -integrin and its heterodimer partner  $\alpha_6$ -integrin, we first performed steady state metabolic labeling followed by immunoprecipitation with either  $\beta_4$ - or  $\alpha_6$ -integrin antibodies or their respective control IgGs (Fig. 1A).  $\beta_4$ -integrin antibodies precipitated a major band at 200 kD (Fig. 1A, left panel, red asterisk) and two additional bands at 150 kD (black asterisk) and 120 kD (blue asterisk).  $\alpha_6$ -integrin antibodies detected two specific bands at a 1:1 stoichiometric ratio, one at 200 kD (Fig. 1A, right panel, red asterisk) and another at 120 kD (Fig. 1A, right panel, blue asterisk). We have identified these bands in a recent study using mass spectroscopy as full-length  $\beta_4$ - ( $\beta_4$ -FL) and  $\alpha_6$ -subunits, respectively (Fig. 1B) [23]. The additional <150 kD fragment, precipitating with an antibody binding to the C-terminus of  $\beta_4$ -integrin, was identified as a truncated form of  $\beta_4$ -integrin ( $\beta_4$ -C) that was lacking most of its extracellular domain. Proteolytic cleavage of  $\beta_4$  from the ectodomain by MMP family of proteases has been reported [24]. To investigate if  $\beta_4$  is similarly processed in MDCK cells by MMPs to release a fragment of the observed size, we treated cells with different concentrations of a broad-spectrum MMP inhibitor. The results indicated that the fragment was indeed sensitive to the inhibitor in a concentration dependent manner, suggesting that  $\beta_4$  ectodomain is generated by MMP-dependent proteolytic cleavage (Fig. S1A).  $\beta_4$ -C did not immunoprecipitate with  $\alpha_6$ -integrin (Fig. 1A). Taking together, these data show that  $\beta_4$  formed a single heterodimer with  $\alpha_6$  in MDCK cells. However, as  $\beta_4$ -integrin is found in large excess over  $\alpha_6$ , only a small fraction of total  $\beta_4$  forms a  $\alpha_6\beta_4$ -heterodimer.

To investigate whether  $\alpha_6\beta_4$  integrins are obligate heterodimers or if  $\beta_4$  can mature independently of  $\alpha_6$ , and vice versa, we generated knockout (KO) cell lines by CRISPR/Cas9-mediated gene editing using a lentivirus-mediated co-expression system for single gRNA constructs and Cas9 [25]. Two independent gRNA constructs were designed for each target gene and puromycin-selected ItgKO cell populations (Fig. S1B)

were screened by western immunoblotting ( $\beta_4$ , Fig. S1C) and immunofluorescence ( $\alpha_6$ , Fig. S1D) for clones that had lost the target protein expression (Fig. S1B). Three verified KO-clones from each construct were selected for further experiments (indicated in Fig. S1C and S1D). Note the disappearance of both  $\beta_4$ -FL and  $\beta_4$ -C bands in  $\beta_4$ KO cells (Fig. 1E).

To study the maturation and cell surface targeting of  $\beta_4$ -integrin, we performed a surface biotinylation assay in control,  $\beta_4$ - and  $\alpha_6$ KO MDCK cells. Specific biotinylation of cell surface proteins was confirmed by western blotting of selected cell surface ( $\beta_4$ -integrin, E-cadherin) and cytoplasmic (Early endosomal antigen 1 (EEA1), calnexin,  $\beta$ -tubulin) proteins from streptavidin precipitations (Fig. 1C). Strikingly, both  $\beta_4$ -FL and particularly the  $\beta_4$ -C could be detected at the surface of  $\alpha_6$ KO cells (Fig. 1D & F). While clones from the  $\alpha_6$ -KO construct 2 ( $\alpha_6$ KO2) expressed significantly lower levels of  $\beta_4$ -FL at the cell surface compared to  $\alpha_6$ KO1, all of them retained normal surface levels of  $\beta_4$ -C and  $\alpha_6$ -KO clone 1 ( $\alpha_6$ KO1) displayed high levels of surface expressed  $\beta_4$ -FL. Moreover,  $\alpha_6$  was abundantly expressed at the surface of both Itg $\beta_4$ -KO cell clones (Fig. 1E & G).

To confirm the surface expression with an independent method we employed total internal reflection fluorescence (TIRF) microscopy that limits the excitation depth to roughly 100 nm from the refractive surface thereby detecting only fluorescence signals that are at, or very close to, the basal surface of the cells. The basal localization of  $\alpha_6$ - and  $\beta_4$ -subunits was analyzed in the different KO cell lines. TIRF analysis in control cells revealed a characteristic hemidesmosome-like elongated staining pattern with antibodies recognizing  $\beta_4$ - or  $\alpha_6$ -integrins (Fig. 1H & I, middle panels). These patches were adjacent but distinct from talin (TLN)-positive focal adhesions (Fig. 1H & I, left panels). In agreement with the surface biotinylation data, the  $\alpha_6$ KO1 exhibited more robust patchy  $\beta_4$ -positive signal at the basal membrane than  $\alpha_6$ KO2 clone, but both of them showed detectable  $\beta_4$ -staining (Fig. 1H–K). In  $\beta_4$ KO cells,  $\alpha_6$ -integrin targeting to the basal surface domain was also reduced but, in addition, the remaining  $\alpha_6$ -staining pattern changed such that it better coincided with talin (Fig. 1I, 1K & L).  $\beta_1$ -integrins and TLNs are central components of actin-linked focal adhesions [26] [27]. Because  $\beta_1$ -integrin was not visible in the  $\alpha_6$ -integrin immunoprecipitations, our data suggests that either free  $\alpha_6$ -subunit can also reach the cell surface or the  $\alpha_6$ -antibody disrupts  $\alpha_6\beta_1$ -integrin heterodimers during immunoprecipitation. Given that  $\alpha_6$ -integrin showed increased co-localization with TLN in  $\beta_4$ -KO cells, we next addressed this issue by generating  $\beta_1/\beta_4$ -dKO cells lacking expression of both  $\beta_1$ - and  $\beta_4$ -integrins and studied the surface levels of  $\alpha_6$ -integrins in these cells (Fig. 1M). This data clearly showed that the surface expression of  $\alpha_6$ -subunit in  $\beta_4$ -KO cells was entirely dependent on heterodimer formation with  $\beta_1$ -subunit (Fig. 1M & N). In the absence of  $\beta_4$ -subunit,  $\alpha_6$ -integrin can be secreted to the plasma membrane as  $\alpha_6\beta_1$ -heterodimer. However,  $\beta_4$ -integrin is required for its localization to putative HDs (Fig. 1I & N). Lateral and basal expression of  $\alpha_6\beta_4$ -integrin was unaffected by depletion of  $\beta_1$ -integrin (Fig. S2B).

Our data showed that  $\beta_4$ -integrin is able to reach the cell surface in the absence of  $\alpha_6$ -integrin. The surface expression of  $\alpha_6$ -subunit was dependent on heterodimer formation with either  $\beta_4$  or  $\beta_1$  (Fig. 1M–N & Fig. S2). Consistent with earlier findings, we found no evidence for a novel  $\beta_4$ -subunit containing integrin heterodimer in  $\alpha_6$ KO cells. This result contrasts the current model according to which  $\beta_4$ -integrin would remain trapped in the ER until it forms a heterodimer with  $\alpha_6$ -integrin. However, it should be noted that previous data supporting an independent HD targeting of both extracellular and intracellular domains of  $\beta_4$ -integrins has been reported [12] [15] [18]. Moreover, a previous study detected a significant amount of monomeric  $\beta_4$ -integrin at the cell surface, although it was suggested to derive from the  $\alpha_6\beta_4$  complex via partial dissociation or due to faster turnover rate of the  $\alpha_6$ -subunit [20]. Based on current results, a significant amount of free, presumably monomeric,  $\beta_4$ -integrin exists at the cell surface also in the absence of  $\alpha_6$ -subunit expression, suggesting that it can be transported to the cell surface as a single subunit. How stable such monomeric  $\beta_4$ -integrin is, remains unknown. The observation that the relative amount of processed  $\beta_4$ -integrin ( $\beta_4$ -C), lacking almost the entire extracellular domain, was increased in  $\alpha_6$ KO cells suggests that monomeric  $\beta_4$ -integrins might be prone to proteolytic processing after which

they are not able to mediate adhesion to laminin. In line with this finding, mouse studies have shown that  $\alpha_6$ -deletion leads to loss of functional HDs and results in lethal fragility in epithelial tissues [28]. However, the processed  $\beta_4$ -C could still promote partial assembly of HD components and help to link other laminin receptor complexes, such as those mediated by  $\beta_1$ -integrins or non-integrin receptors like the dystroglycan, to the IF network. Further studies are warranted to explore these possibilities.

## Conclusions

In conclusion, our data shows that free  $\beta_4$ -integrin can be targeted to the cell surface where it forms HD-like structures also in the absence of  $\alpha_6$ -subunit, although with visibly reduced efficiency. The ectodomain of surface expressed  $\beta_4$ -integrins can be proteolytically cleaved and this process is enhanced in  $\alpha_6$ -KO cells. Nevertheless, steady-state biosynthetic labeling experiments confirmed that significant levels of full-length free  $\beta_4$ -integrins are expressed at the cell surface. Whether this monomeric  $\beta_4$ -integrin is signaling-competent and has independent cellular functions remains to be studied.

## Limitations

The current study is based on in vitro cell culture model. Whether  $\beta_4$ -integrin can be found at cell surface in, for example,  $\alpha_6$ -integrin-deficient mice, remains to be studied.

## Alternative Explanations

## Conjectures

The initial findings should be followed by studies addressing the signaling potential of  $\beta_4$ -integrin in  $\alpha_6$ -deficient cells.

## Additional Information

### Methods

#### Antibodies and Reagents

Primary antibodies (see also Table S1) targeting  $\beta_1$ -integrin were purchased from Thermo Fischer Scientific (TS2/16; #MA2910). Anti- $\beta_4$ -integrin antibodies were purchased from Santa Cruz (sc-6628) and anti- $\alpha_6$ -integrin (555734-GoH3) and anti-E-cadherin (610181) antibodies were from BD Biosciences. Anti-LN- $\gamma_1$  (L9393), anti- $\beta$ -actin (A5441), anti- $\beta$ -tubulin (T4026) and anti-talin (T3287) antibodies were from Sigma-Aldrich. Anti-Calnexin (ab10286) and anti-EEA1 (ab2900) antibodies were from Abcam. Peroxidase-conjugated secondary antibodies for western immunoblotting and fluorophore-conjugated secondary antibodies for immunofluorescence stainings were purchased from Jackson ImmunoResearch. Goat and rat total IgGs, used as negative controls in immunoprecipitations, were also from Jackson ImmunoResearch. Alexa Fluor 488 phalloidin from Invitrogen was used for staining of F-actin and DAPI from Sigma-Aldrich was used for staining of nuclei. Surface-biotinylated and immunoprecipitated proteins were detected from western immunoblots using peroxidase streptavidin (Jackson ImmunoResearch).

#### Cell culture and Treatments

MDCK-II cells (ATCC:CCL-34) were routinely cultured in 5% fetal bovine serum (Invitrogen) containing minimal essential medium (MEM, Invitrogen) with 1% penicillin and streptomycin. For analysis of matrix adhesions in confluent cells, cells were seeded onto

coverslips or glass bottom dishes (for TIRF microscopy) and cultured in serum containing medium for 6 days. When indicated, cells were cultured in the presence of GM6001 (Sigma-Aldrich) at different concentrations for 24 h.

### **KO of integrins via lentivirus-mediated expression sgRNAs and Cas9**

Gene editing with lentivirus-mediated co-expression of Cas9 and sgRNA-constructs was done with some modifications as previously described [25]. Second exon of canine ITGA6 and fourth exon of canine ITGB4 were used as a template for gRNA-design. Target sequences with no off-target sites with less than 3 mismatches in the canine genome database (European Nucleotide Archive), were selected based on FASTA similarity search tool (EMBL-EBI; Table S2). Two targeting sequences for each gene were selected and the corresponding gRNA oligos with BsmBI overhangs were subcloned into lentiCRISPRv1 ( $\beta_4$ - and  $\alpha_6$ -KOs; Addgene #49535) or lentiCRISPRv2 ( $\beta_1$ -KOs; Addgene #52961, [29]).

For generation of the lentiviruses, 70–80% confluent 293T cells on CellBind 10 cm dishes (Corning) were co-transfected with lentiCRISPRv1 (20  $\mu$ g), pPAX2 (15  $\mu$ g) and VSVg (5  $\mu$ g) by Lipofectamine 2000 reagent (Invitrogen) in OptiMEM (Invitrogen). Medium was collected over a period of 24 h to 96 h post-transfection in 12 h patches. Virus-containing medium was pooled and filtered through a 0.44  $\mu$ m filter, followed by pelleting of the virus by ultracentrifugation at 100,000  $\times$  g for 2 h at 4°C. 1/10th dilution of the 100X virus concentrate was used for infection of MDCK cells seeded at  $6 \times 10^4$ /24-well, 24 h prior. 24 h post-infection, virus-containing media was exchanged with normal media to allow cells to recover. 48 h post-infection, cells were trypsinized and re-seeded in the presence of 6  $\mu$ g/ml puromycin, followed by 24 h of selection. Puromycin-resistant cells were analyzed for KO frequency using immunofluorescence staining (Fig. S1B). Clonal cell populations were generated and analyzed for loss of protein expression as shown in figure S1C and S1D. Three clonal cell lines for each gRNA construct were generated and used in replicate experiments.

### **Cell surface biotinylation**

Cell surface biotinylation was adapted from [30]. Cells were seeded at a density of  $4.5 \times 10^4$ /cm<sup>2</sup> onto 10 cm tissue culture dishes 24 h prior. Cells were washed thrice with biotinylation buffer (20 mM HEPES, 130 mM NaCl, 5 mM KCl, 0.8 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub>), followed by biotinylation with 0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce) in biotinylation buffer 30 min on ice with gentle rotation. Cells were washed three times with 10 mM Tris-HCl, 0.15 M NaCl, pH 7.45, followed by lysis with RIPA buffer (10 mM Tris-HCl, 0.15 M NaCl, 0.5% SDS, 1% IGEPAL, 1% sodium deoxycholate) supplemented with protease inhibitors (Roche).

### **Immunoprecipitation and Avidin-precipitation**

RIPA-lysates were rotated 30 min +4°C with Benzonase nuclease (Novagen) and filtered through a 0.45  $\mu$ m Spin-X column (Corning). Protein concentration was determined with the bicinchoninic acid assay (Pierce). For analysis of surface biotinylated integrins, lysates with 150  $\mu$ g total protein were incubated o/n at +4°C with 3  $\mu$ g of primary antibodies or control IgGs, and immunoprecipitated with 1.5 mg of Protein-G Dynabeads (Invitrogen) or 30  $\mu$ l of protein-G agarose beads (Pierce) for 2–3 h +4°C. Beads were washed thrice with RIPA buffer and once with 10 mM Tris-HCl, pH 6.8. For analysis of metabolically labelled integrins, 200–300  $\mu$ g of total protein was incubated with 5–7.5  $\mu$ g of primary antibodies or control IgGs and processed as above. Beads were cooked with 2X Laemmli sample buffer with 2%  $\beta$ -mercaptoethanol 4 min at +97°C. Cell surface biotinylated cell lysates were avidin-precipitated with avidin agarose beads (Pierce).

### **SDS-PAGE and Western immunoblotting**

RIPA-lysates were prepared into 1X Laemmli sample buffer with 1%  $\beta$ -mercaptoethanol and cooked 4 min at +97°C. Proteins were separated in 6% SDS-PAGE and blotted o/n at +4°C at 20V in with 20% ethanol in 0.025 M Tris 0.192 M Glycine onto a nitrocellulose membrane (Perkin-Elmer). Membranes were reacted with primary antibodies o/n at +4°C, followed by incubation with peroxidase-conjugated secondary antibodies (Jackson

Immunoresearch). Antibody bands were detected with the Lumi-Light chemiluminescence kit (Roche) with the LAS-3000 imager. Protein bands were quantified with the Quantity One software (Biorad) and relative levels of KOs were determined either by directly dividing from control or from a standard curve prepared from serially diluted control.

### Metabolic labeling and Autoradiography

Metabolic labeling protocol was adapted from [31]. Briefly, cells were seeded as  $4 \times 10^5$ /cm onto 6 cm  $\varnothing$  tissue culture dishes and cultured for 3 days followed by 18 h of labeling at  $+37^\circ\text{C}$  with 100  $\mu\text{Ci}$  of EasyTag™ EXPRE35S35 Protein Labeling Mix (PerkinElmer) in medium containing 1/10th normal concentration of methionine and cysteine. Labelled cells were washed three times with cold PBS and lysed in 1 ml of RIPA buffer on ice. For analysis of metabolically labelled integrins, 200–300  $\mu\text{g}$  of total protein was incubated with 5–7.5  $\mu\text{g}$  of primary antibodies or unspecific IgGs. Immunoprecipitation and SDS-PAGE were performed as described above. Gels were de-stained for 10 min at room temperature (RT) with 45% methanol and 10% acetic acid) and impregnated with Kodak™ ENLIGHTNING™ Rapid Autoradiography Enhancer (PerkinElmer) for 30 min at RT in the dark. Gels were dried and exposed onto BioMax® XAR films (Carestream).

### Immunofluorescence

Cells were fixed with 4% PFA in PBS+/+ (PBS with 0.5 mM  $\text{MgCl}_2$  and 0.9 mM  $\text{CaCl}_2$ ) for 15 min at RT. After quenching for 20 min with 0.2 M glycine in PBS, cells were permeabilized with 0.1% TX-100 in PBS for 10 min. All subsequent steps on saponin-permeabilized cells contained 0.02% saponin. Permeabilized cells were blocked with 0.5% BSA in PBS for minimum of 30 min and primary antibodies were prepared in blocking buffer and incubated with cells o/n at  $+4^\circ\text{C}$ . After 1 h incubation with secondary antibodies at RT, cells were mounted with Immu-Mount (company). When used, DAPI and phalloidin dyes were prepared with secondary antibodies. For staining with PANCytokeratin antibody, cells were fixed with 1:1 mixed methanol and acetone at  $-20^\circ\text{C}$  and quenching and permeabilization steps omitted.

### Microscopy and Image analysis

Cells were seeded onto glass coverslips (for confocal microscopy) or glass-bottom dishes (for TIRF) and cultured to confluency. For immunofluorescence staining, cells were fixed with 4% PFA in PBS+/+ (PBS with 0.5 mM  $\text{MgCl}_2$  and 0.9 mM  $\text{CaCl}_2$ ) for 15 min at RT. After quenching for 20 min with 0.2 M glycine in PBS, cells were permeabilized with 0.1% TX-100 in PBS for 10 min. Permeabilized cells were blocked with 0.5% BSA in PBS for minimum of 30 min and primary antibodies, were prepared in blocking buffer and incubated with cells overnight at  $+4^\circ\text{C}$ . After 1 h incubation with secondary antibodies at RT, cells were mounted with Immu-Mount™ (Thermo Fisher Scientific). When used, DAPI and phalloidin dyes were prepared with secondary antibodies. Confocal images were acquired with the Zeiss LSM-780 laser scanning confocal microscope using 40X Plan-Apochromat objective (N.A = 1.4) and TIRF images with the Zeiss Cell Observer spinning disc confocal microscope using the alpha Plan-Apochromat 63X oil objective with an N.A of 1.46. For TIRF image acquisition, immunofluorescence stained samples in glass bottom dishes were left unmounted and were kept in PBS. Co-localization in TIRF images was assessed with the Pearson's correlation coefficient measured with the Co-localization Threshold plugin in FIJI using Costes method auto threshold determination and excluding zero intensity pixels. Unthresholded PCC values were used in the analysis due to the high labelling density of the matrix staining, which interferes with the thresholding algorithm. One channel was rotated  $90^\circ$  degrees relative to the other channel and the misaligned images analyzed to demonstrate the absence of random correlation.

### Statistics

Absolute values were tested for significant differences with one-way analysis of variance (ANOVA) using Tukey's post-hoc test. Fold changes were tested for significant



differences with two-tailed one-sample t-test. All statistical analyses were carried out with SPSSv20.

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### **Ethics Statement**

Not applicable

## Citations

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